JOURNAL OF VIROLOGY, Jan. 1999, p. 474–481 Vol. 73, No. 1
0022-538X/99/$04.00+0

Hepatitis B Virus RNA-Binding Proteins Associated with Cytokine-Induced Clearance of Viral RNA from the Liver of Transgenic Mice†

TILMAN HEISE,[1,2] LUCA G. GUIDOTTI,[1] VICTORIA J. CAVANAUGH,[1] AND FRANCIS V. CHISARI[1]*

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037,[1] and Institut für Biochemie und Molekulare Zellbiologie, Georg-August-Universität Göttingen, D-37073 Göttingen, Germany[2]

Received 20 July 1998/Accepted 7 October 1998

Hepatitis B virus (HBV) gene expression is downregulated in the liver of HBV transgenic mice by a posttranscriptional mechanism that is triggered by the local production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) during intrahepatic inflammation (hepatitis). The molecular basis for this antiviral effect is unknown. In this study, we identified three HBV RNA-binding liver nuclear proteins (p45, p39, and p26) the relative abundance of which correlates with the abundance of HBV RNA in response to the induction of IFN-γ and TNF-α. All three proteins bind to a 91-bp element located at the 5′ end of a previously defined posttranscriptional regulatory element that is thought to mediate the nuclear export of HBV RNA. The presence of p45 correlates directly with the presence of HBV RNA, being detectable under baseline conditions when the viral RNA is abundant and undetectable when the viral RNA disappears in response to IFN-γ and TNF-α. In contrast, p26 is inversely related to HBV RNA, being detectable only when the viral RNA disappears following cytokine activation. Finally, p39 is constitutively expressed, and its abundance and mobility appear to be slightly increased by cytokine activation. These results suggest a model in which hepatocellular HBV RNA content might be controlled by the stabilizing and/or destabilizing influences of these RNA-binding proteins whose activity is regulated by cytokine-induced signaling pathways.

Hepatitis B virus (HBV) is a noncytopathic, hepatotropic virus with a 3.2-kb circular DNA genome that encodes four overlapping 3.5-, 2.4-, 2.1-, and 0.7-kb unspliced messages that terminate at a common polyadenylation site (51). Because HBV does not replicate in tissue culture or in genetically or immunologically defined animals, the development of an HBV transgenic mouse model was required to define the host-virus interactions involved in viral clearance and disease pathogenesis (2, 14, 16, 28, 44). Based on these studies, it is now clear that the vigor and kinetics of the cellular immune response to HBV, especially the cytotoxic T-lymphocyte (CTL) response, determines the outcome of HBV infection (15).

Using this model, we demonstrated that, in addition to killing HBV-positive hepatocytes, HBV-specific CTLs can downregulate hepatocellular HBV gene expression and replication by a noncytopathic, cytokine-induced process that is mediated by inflammatory cytokines such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) secreted by the CTLs following antigen recognition in the liver (27). In addition, we showed that HBV gene expression and replication are downregulated noncytopathically during lymphocytic choriomeningitis virus (LCMV) (25)- and murine cytomegalovirus (MCMV) (8)-induced hepatitis in these animals. By nuclear run-on analysis, we showed that these cytokines downregulate HBV gene expression posttranscriptionally, since the viral transcription rate is virtually unchanged following cytokine induction despite the absence of detectable viral RNA (60). Those results confirmed previous studies demonstrating that recombinant TNF-α (23) and interleukin-2 (IL-2) (29) downregulate hepatocellular HBV mRNA in a lineage of transgenic mice in which HBV gene expression is controlled by the metallothionein promoter, despite the fact that the endogenous metallothionein mRNA was upregulated by the cytokines in the same tissues. The intracellular mechanisms whereby these inflammatory cytokines posttranscriptionally destabilize HBV RNA remain to be determined.

RNA-protein interactions play an important role in the regulation of splicing (54), nuclear export (35), stabilization (49), and destabilization (17, 48, 52) of cellular mRNA. In the systems studied thus far, cellular RNA-binding proteins and RNases influence transcript stability by interacting with sequence and/or structural elements in the RNA. For example, short-lived mRNAs such as c-*fos* and granulocyte-macrophage colony-stimulating factor mRNAs contain AU-rich sequences in their 3′ untranslated regions that interact with various RNA-binding proteins (12), including the AU-rich binding factor (AUF) (6) and the adenosine-uridine-binding protein (41) that destabilize the mRNA (12, 13, 55). AUF is also part of a protein complex (α-complex) that stabilizes globin mRNA (36, 62). Furthermore, the transferrin receptor mRNA is posttranscriptionally regulated by the interaction of iron response elements (IRE) in the RNA with an IRE-binding protein (42) whose binding activity, which is induced by low cellular iron concentrations (31) and phosphorylation (20), protects the transferrin receptor mRNA from endonucleolytic cleavage (4). Additionally, the nuclear export of unspliced human immunodeficiency virus (HIV) mRNA requires the interaction between a viral RNA sequence, the Rev response element (RRE), and the HIV Rev protein which, together with host

* Corresponding author. Mailing address: Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 784-8228. Fax: (619) 784-2160. E-mail: fchisari@scripps.edu.

† Manuscript no. 11630-MEM from The Scripps Research Institute.

factors, facilitates the export of the HIV RNA into the cytoplasm (21).

Recently, we showed that the 0.7-kb HBV transcript, which overlaps the 3′ untranslated regions of all of the longer HBV transcripts, is resistant to cytokine-induced destabilization (60) whereas the longer transcripts are suppressed, suggesting that one or more elements located between nucleotides (nt) 3157 and 1239, upstream of the start site of the 0.7-kb mRNA and downstream of the 2.1-kb transcript start site, are required for cytokine-induced destabilization of the 2.1-, 2.4-, and 3.5-kb mRNAs. At least two elements which could serve as targets for cellular RNA-binding proteins are present in this region. The first is an AU-rich region (nt 767 to 870) containing one copy of the destabilizing AUUUA element found in short-lived RNAs (12, 13, 55). The second is a previously identified posttranscriptional regulatory element (PRE) located between nt 1239 and 1805 which is thought to be required for nuclear export of unspliced HBV RNA (18, 32, 33). Recently, cellular proteins (p30 and p45) that interact with this element and might be part of an HBV RNA export mechanism have been identified (34).

Based on these observations, we thought to identify and characterize cellular HBV RNA-binding proteins that might contribute to the posttranscriptional regulation of HBV RNA by binding to a 91-bp sequence located in the HBV PRE immediately upstream of the cytokine-resistant 0.7-kb HBV mRNA but within the cytokine-sensitive viral transcripts. In UV cross-linking experiments, we identified three HBV RNA-binding proteins (p45, p39, and p26) that bind to this element in liver nuclear extracts from untreated, CTL-injected, MCMV-infected, and LCMV-infected HBV transgenic mice. We showed that the cytokine-induced downregulation of HBV RNA in these experiments was tightly associated with the disappearance of p45 and the appearance of p26. The strong association between these RNA-binding proteins and intrahepatic HBV RNA content suggests that they may be part of a complex mechanism mediating the destabilizing effects of IFN-γ and TNF-α on HBV gene expression in this model.

MATERIALS AND METHODS

HBV transgenic mice. The HBV transgenic mouse lineages 219 (official designation, pFC80-219), 1.3.32 (official designation, Tg{HBV 1.3 genome}Chi32), and 1.3.46 (official designation, Tg{HBV 1.3 genome}Chi46) used in this study have been described previously (23, 28). Lineage 219 expresses high levels of the HBV envelope 2.1-kb mRNA in the vast majority of hepatocytes under transcriptional control of the HBV surface promoter (26). Lineages 1.3.32 and 1.3.46 express all of the viral RNAs under the control of the respective viral promoters, and they replicate HBV at high levels in the liver and kidney without any evidence of cytopathology (28). Mice were matched for age (8 to 10 weeks), sex (male), and serum hepatitis B e antigen concentration by using a commercially available solid-phase radioimmunoassay (Sorin Biomedica, Saluggia, Italy).

HBsAg-specific CTLs. An L^d-restricted, $CD3^+$ $CD4^-$ $CD8^+$ hepatitis B surface antigen (HBsAg)-specific CTL clone, designated 6C2, was used for this study. These CTLs recognize an epitope located between residues 28 and 39 of HBsAg (HBsAg28-39), and they secrete IFN-γ and TNF-α upon recognition of antigen (2). In all experiments, 10^7 CTLs were injected intravenously into transgenic mice 5 days after in vitro stimulation with irradiated P815 cells that stably express the HBV large envelope protein (2). CTL-induced liver disease was monitored by measuring serum alanine aminotransaminase activity at various time points after CTL injection. Aliquots of liver tissue obtained at autopsy were either fixed in zinc formalin, embedded in paraffin and processed for histological and immunohistochemical analysis, or snap-frozen in liquid nitrogen for subsequent molecular analyses.

IL-12 injection. Recombinant murine IL-12 (Hoffmann-La Roche, Nutley, N.J.) was injected (100 ng daily for 3 consecutive days) intraperitoneally into HBV transgenic mice (lineage 1.3.46). The mice were sacrificed 16 h after the last injection, and their livers were harvested and processed as described elsewhere (9). Total liver RNA and nuclear extracts were prepared from the same livers.

LCMV. HBV transgenic mice (lineage 1.3.32) were infected by intravenous inoculation of 2×10^6 PFU of LCMV WE clone 2.2 (25), and measurements of serum and liver LCMV titers as well as histological and molecular analyses for hepatic HBV RNA were performed on zinc-formalin-fixed and snap-frozen tissue as previously described (25). Total liver RNA and nuclear extracts were prepared from the same livers.

Adenovirus infection. A recombinant, replication-deficient adenovirus (Ad.C-BlacZ [38]) was used to infect mice (lineage 1.3.32) at various doses (1.5×10^9 or 5.0×10^9) via the tail vein as previously described (8). Animals were sacrificed at multiple time points following infection, and their livers were harvested and either processed for histological analysis or snap-frozen in liquid nitrogen and stored at −80°C for subsequent molecular analyses as previously described (8).

MCMV infection. The Smith strain of MCMV (ATCC VR-194; American Type Culture Collection, Rockville, Md.) was used in this study. Lineage 1.3.32 mice were injected intraperitoneally with 5×10^4 PFU of MCMV (8) and sacrificed at various time points thereafter. Livers were harvested, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent molecular analyses as previously described (8).

RNA analyses. Frozen liver tissues were mechanically pulverized, and total genomic RNA was isolated for Northern blot analysis and for RNase protection assay exactly as described elsewhere (27, 28).

Anticytokine antibodies. Hamster monoclonal antibodies H22 and TN3 19.12, specific for murine IFN-γ and TNF-α (53, 56) and generously provided by Robert Schreiber (Washington University, St. Louis, Mo.), were used in this study. Purified hamster immunoglobulin G (Jackson ImmunoResearch, West Grove, Pa.) was used as the control antibody. All antibodies were diluted to a concentration of 1,250 μg per ml with nonpyrogenic phosphate-buffered saline (GIBCO BRL, Gaithersburg, Md.) immediately before injection, and 250-μg doses of the antibodies were administered to animals intraperitoneally 24 h before and 2 days after the intravenous injection of CTLs.

Preparation of nuclear extracts. Frozen mouse or human liver tissue (0.2 to 0.5 g), 8×10^7 partially purified (~60% pure as determined by light microscopy) hepatocytes from HBV transgenic mice lineage 1.3.46, or 10^7 cryopreserved HepG2 cells were thawed in a fivefold volume of ice-cold homogenization buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA (buffer A), 0.5 mM dithiothreitol (DTT), and 1/25 volume of proteinase inhibitor mix (Boehringer Mannheim, Indianapolis, Ind.) and homogenized by five strokes in a glass homogenizer with a loose-fitting motor-driven (50 rpm) Teflon pestle. The homogenate was centrifuged at $2,000 \times g$ for 20 min; the resulting supernatant was stored at −80°C. The pellet was resuspended in 6 ml of buffer containing buffer A and 0.88 M sucrose (buffer B), loaded on a 7-ml cushion of buffer B, and centrifuged at $10,000 \times g$ for 30 min. The supernatant was discarded, and the pellet was dissolved in 5 ml of buffer containing buffer A and 2.0 M sucrose (buffer C). The slurry was loaded on a 7-ml cushion of buffer C and centrifuged at $180,000 \times g$ for 70 min. The supernatant was discarded, and the nuclei were resuspended in 100 μl of storage buffer containing 20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 50% glycerol, 0.5 mM DTT, and 1/10 volume of proteinase inhibitor mix (Boehringer Mannheim). Nuclei were counted by light microscopy and lysed by adding 5× lysis buffer containing 100 mM Tris-HCl (pH 8.0), 2.1 M NaCl, 7.5 mM $MgCl_2$, 1.0 mM EDTA, and 25% glycerol to final concentrations of 33 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT, and 1/10 volume proteinase inhibitor mix (Boehringer Mannheim). The viscous lysate was transferred into dialysis tubes (molecular weight cutoff, 6,000 to 8,000; Spectro/Por; Spectrum Companies, Gardena, Calif.) and dialyzed three times against 500 ml of dialysis buffer F containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and proteinase inhibitor Mix (Boehringer Mannheim). The dialyzed nuclear extract was cleared by centrifugation for 10 min at $25,000 \times g$, and the protein content was determined by the Bradford dye binding procedure, using a commercial kit (Bio-Rad Laboratories, Hercules, Calif.).

In vitro transcription. A plasmid containing the entire HBV genome (*ayw* subtype) was used for the production of DNA templates for the generation of HBV transcripts. Two primers were used. Primer 1 (5′-CC*ATCGAT*-**TAATACG ACTCACTATAG**-3′) contained a restriction site for *Cla*I (shown in italics), the T7 RNA polymerase promoter sequence (shown in boldface), and the sense HBV *awy* DNA sequence (22) (accession no. J02203) spanning nt 1243 to 1259; primer 2 contained antisense HBV sequence from nt 1312 to 1333. Transcripts spanning nt 3161 to 1988, 3161 to 870, 3161 to 409, 767 to 870, 767 to 925, 987 to 1988, 987 to 1124, 987 to 1302, 987 to 1491, 987 to 1775, 1530 to 1988, 1530 to 1786, 1530 to 1775, 1242 to 1491, 1242 to 1410, 1242 to 1775, 1242 to 1786, and 1242 to 1988 were produced and tested for binding (not shown). The mouse β-actin template was generated by using the following primers. Primer 1 included a *Cla*I site and a T7 RNA polymerase promoter followed by β-actin-specific DNA sequence spanning nt 27 to 45 (1); primer 2 contained antisense β-actin-specific sequence between nt 121 and 140 (1). The mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template was generated by using the following primers. Primer 1 included a *Cla*I site and a T7 RNA polymerase promoter followed by GAPDH-specific DNA sequence spanning nt 383 to 401 (50); primer 2 contained antisense GAPDH-specific sequence between nt 478 and 497 (50).

The HIV RRE template was generated by using the following primers. Primer 1 (5′-GAGCAGTGGGAATAGTAGG-3′) included a ClaI site and a T7 RNA polymerase promoter followed by RRE-specific DNA sequences; primer 2 (5′-TCCCTAGGAGCTGTTGAT-3′) contained antisense RRE-specific sequence.

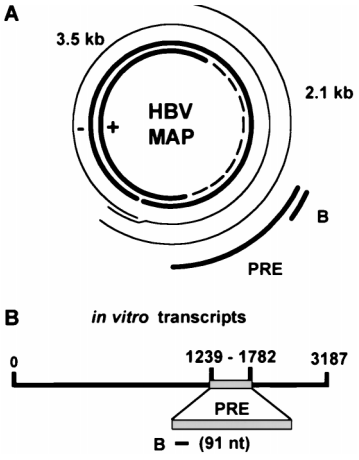

FIG. 1. (A) Schematic map of the HBV genome showing the 3.5-kb pregenomic RNA and the 2.1-kb envelope RNA. (B) Location of the PRE (nt 1239 to 1805) (34) and position of in vitro RNA-B (nt 1243 to 1333) used in this study.

The Mason-Pfizer virus constitutive transport element (CTE) template was generated by using the following primers. Primer 1 included a *Cla*I site and a T7 RNA polymerase promoter followed by Mason-Pfizer virus-specific DNA sequence spanning nt 8007 to 8025 (57); primer 2 contained antisense Mason-Pfizer virus-specific sequence between nt 8221 and 8238 (57). PCRs for HBV, RRE, and CTE templates were produced with 1 ng of plasmid (plasmids containing specific RRE and CTE sequences were a generous gift from T. J. Hope). PCRs for β-actin and GAPDH templates were produced with reverse-transcribed mouse liver RNA, the mixture contained 80 pmol of each primer in 1× PCR buffer, 0.2 mM GTP, ATP, TTP, and CTP, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). PCR was performed as follows: 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 56°C, 1 min at 72°C, and 5 min 72°C). The PCR products were purified by size exclusion using a commercial kit (PCR purification kit; Boehringer Mannheim), ethanol precipitated, and used as templates to generate transcripts. Transcription reactions were carried out with 0.5 to 1.0 μg of PCR product in a final volume of 20 μl in transcription buffer (Promega, Madison, Wis.) containing 0.31 mM ATP, CTP, and GTP and 7.5 mM [α-^{32}P]UTP (800 Ci/mmol) (NEN, Boston, Mass.), 5 mM DTT, and 20 U of RNasin (Promega). The reaction was started by addition of 20 U of T7 RNA polymerase (Promega). After incubation for 45 min at 37°C, another 20 U of T7 RNA polymerase was added and the reaction was continued for 45 min at 37°C. The reaction was terminated by adding 10 μg of yeast tRNA and 1 U of DNase I (Promega), and the mixture was incubated for 15 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, transcripts were dissolved in 10 mM Tris-HCl (pH 7.4)–diethyl pyrocarbonate-treated water.

UV cross-linking experiments. Standard binding reactions were carried out in a final volume of 40 μl with 5 μg of total nuclear protein and 40 fmol of the ^{32}P-labeled HBV transcript B in binding buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 1.5 mM EDTA, 450 mM NaCl, 0.01% Triton X-100, 20 μg of yeast tRNA, and 6 μg of heparin for 20 min at room temperature. The reaction mixtures were incubated on ice and irradiated for 10 min with UV light (254 nm) in a Stratalinker (Stratagene, La Jolla, Calif.) approximately 3 cm under the bulbs and then digested with 40 μg of RNase A and 100 U of RNase T_1 for 45 min at 37°C; 40 μl of sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 5% mercaptoethanol, 63 mM Tris-HCl [pH 6.8], 10% glycerol, 0.01% bromophenol blue) was added; samples were boiled for 5 min, placed on ice, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% gels. After electrophoresis, the gels were stained with Coomassie blue, destained, dried, and exposed to Kodak Biomax (Kodak, Rochester, N.Y.) overnight at −80°C. Competition experiments were carried out by addition of excess cold competitor to the binding reaction mixture 3 min before or after addition of the labeled transcript.

RESULTS

Identification of three liver nuclear proteins with HBV RNA-binding activity. UV cross-linking was performed to identify HBV RNA-binding proteins that might be involved in the downregulation of HBV RNA after CTL injection. Liver nuclear extracts obtained from HBV transgenic mice after the injection of saline or HBsAg28-39-specific CTLs were analyzed for the ability to bind a series of ^{32}P-labeled in vitro transcripts located between the start sites of the 2.1- and 0.7-kb HBV mRNAs (see Materials and Methods). Because reproducible binding was obtained with a 91-nt transcript representing nt 1243 to 1333, all subsequent experiments were performed with this transcript (henceforth designated RNA-B) (Fig. 1).

In liver nuclear extracts from saline-injected HBV transgenic mice from lineage 219, whose liver contains abundant quantities of the 2.1-kb HBV RNA (Fig. 2A, upper panel), two distinct RNA-protein complexes of 45 and 39 kDa (p45 and p39) were detectable with RNA-B (Fig. 2A, lower panel, lane 1). In contrast, p45 was undetectable and a new 26-kDa RNA-protein complex (p26) appeared in liver nuclear extracts from HBV transgenic mice sacrificed 5 days after CTL injection, coincident with the disappearance of HBV RNA (Fig. 2A, lanes 2 to 4). In addition, the signal intensity and the electrophoretic mobility of p39 are slightly increased under these conditions (Fig. 2A).

To confirm and extend these observations, we examined the ability of liver nuclear extracts from an independent lineage of transgenic mice (1.3.32) to bind RNA-B before and after CTL injection. This lineage expresses all of the viral RNAs (28), including the 2.1-kb HBV mRNA and the overlapping 3.5-kb mRNA which are easily detectable by Northern blot analysis of total liver RNA (Fig. 2B). Once again, p45 and p39 were detectable in the liver before CTL injection, coincident with baseline levels of HBV RNA (Fig. 2B, lane 1), and p45 was strongly reduced following CTL injection, coincident with the appearance of p26 and the disappearance of HBV RNA from the same specimens (Fig. 2B, lane 2). All of these events were blocked by the prior injection of TNF-α- and IFN-γ-specific antibodies (Fig. 2B, lanes 3 to 5) which we have previously reported to prevent the CTL-induced downregulation of HBV RNA (27). Thus, it appears that IFN-γ and TNF-α modulate the balance of HBV RNA-binding proteins as well as the abundance of HBV RNA in the liver. Since RNA-binding proteins are known to influence the stability of cellular mRNAs (49), and since downregulation of HBV gene expression

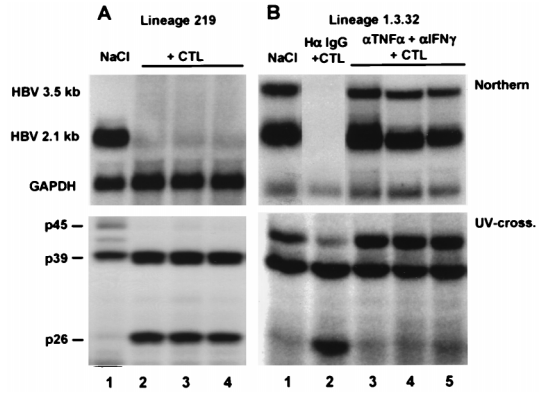

FIG. 2. HBV RNA-binding protein p45 is detectable primarily in liver nuclear extracts from untreated mice, while p26 is detectable mainly in liver nuclear extracts from CTL-injected mice. Northern blot (top) and UV cross-linking (bottom) analyses of 20 μg of total liver RNA or 5 μg of liver nuclear extracts isolated and prepared from the same liver of HBV transgenic mice lineages 219 and 1.3.32 were performed as described in Materials and Methods. (A) Three sex- and serum HBsAg-matched mice (lineage 219) were intravenously injected with 10^7 CTLs and sacrificed at 5 days later. Results were compared with those for a mouse that was sacrificed 5 days after saline injection. (B) Three sex- and serum HBsAg-matched lineage 1.3.32 mice were intraperitoneally injected with 250 μg of hamster monoclonal antibodies against TNF-α and IFN-γ 24 h before and 2 days after intravenous injection of 10^7 HBsAg-specific CTLs. Two control mice were injected either with saline or with 250 μg irrelevant anti-hamster immunoglobulin G (Hα IgG). Mice were sacrificed on day 5 after CTL administration.

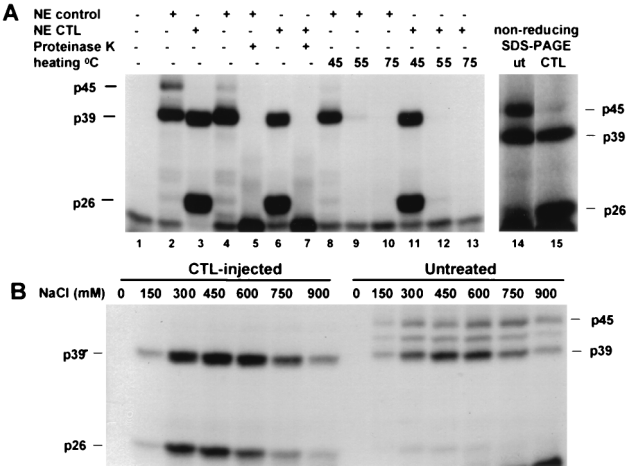

FIG. 3. Characteristics of RNA-binding proteins p45, p39, and p26. Standard UV cross-linking experiments were performed under conditions described in Materials and Methods. Liver nuclear extracts (NE) from untreated (ut; 3 μg) or CTL-injected (2 μg) HBV transgenic mice were incubated with 40 fmol of ^{32}P-labeled RNA-B. (A) Pretreatment of extracts with proteinase K (20 μg) was performed for 30 min at 37°C, or extracts were heated at 45°, 55°, and 75°C for 10 min before the binding reaction. UV cross-linking samples in lanes 14 and 15 were analyzed under nonreducing conditions. (B) Nuclear extracts from untreated (2 μg) or CTL-injected (2 μg) HBV transgenic mice were incubated with 40 fmol of ^{32}P-labeled RNA-B without NaCl or at increasing concentrations of NaCl as indicated.

in both of these lineages is a posttranscriptional CTL-induced process mediated by IFN-γ and TNF-α (27, 60), these results suggest that HBV mRNA stability may be controlled by the CTL- or cytokine-regulated RNA-binding proteins shown in Fig. 2.

Characteristics and specificity of nuclear HBV RNA-binding activities. As shown in Fig. 3A, all three RNA-binding proteins are proteinase K and temperature sensitive, indicating their protein content. p45 is especially temperature sensitive, since its activity also decreases after overnight incubation at 37°C and it progressively disappears after repeated freeze-thaw cycles, while the other proteins are unaffected by these conditions (not shown). The same pattern of RNA-protein complexes was observed when SDS-PAGE was performed under nonreducing conditions, indicating that p45, p39, and p26 are not covalently bound to each other (Fig. 3A, lanes 14 and 15). Further analysis revealed that the proteins do not require divalent cations for binding and that binding is inhibited at low temperature and acid pH (not shown). Based on these observations, all subsequent experiments were performed in the presence of 3 mM $MgCl_2$–0.5 mM EDTA at 23°C and pH 7.4. Under these conditions, as shown in Fig. 3B, the RNA-protein interaction is optimal at high salt concentrations (450 mM NaCl for p39 and p26; 600 mM NaCl for p45), indicating that nonelectrostatic interactions may contribute to their interaction with RNA-B. Finally, we demonstrated that RNA binding by all three proteins is a rapid event, with maximal complex formation occurring within 2.5 min after RNA-B is added to the nuclear extracts (not shown). Since the binding reaction was followed by 10 min of UV irradiation, a total of 12.5 min may actually be necessary for maximal complex formation in vitro.

Competition experiments were performed to study the specificity of the HBV RNA-binding proteins for the 91-nt element used in the UV cross-linking experiments. The binding of p45, p39, and p26 to RNA-B could be competed in a concentration-dependent manner by a 10- to 60-fold excess of unlabeled RNA-B (Fig. 4A, upper panel) but not by the same molar excess of unrelated HBV transcripts (nt 767 to 874) or transcripts containing GAPDH, HIV RRE, or Mason-Pfizer virus CTE sequence (not shown; see Materials and Methods) or by up to a 1,500-fold molar excess of an actin transcript (Fig. 4A, lower panel). These data indicate that recognition of HBV RNA-B by p45, p39, and p26 is relatively selective. In related experiments to be reported separately, we have shown that all three proteins bind to a predicted stem-loop structure located between nt 1275 and 1281 within transcript B (29a).

All of the preceding experiments were performed with total mouse liver nuclear extracts. To be relevant to the posttranscriptional control of HBV RNA, these RNA-binding proteins must be present in hepatocytes, and most importantly in human hepatocytes. As shown in Fig. 4B, not only are p45 and p39 present in partially purified mouse hepatocytes, but nuclear extracts prepared from normal human liver and from the HepG2 human hepatoma cell line also contain similar, although slightly larger, HBV RNA-binding proteins (Fig. 4B). Additional studies will be required to determine whether these human liver-derived RNA-binding proteins are homologs of the proteins detected in the mouse.

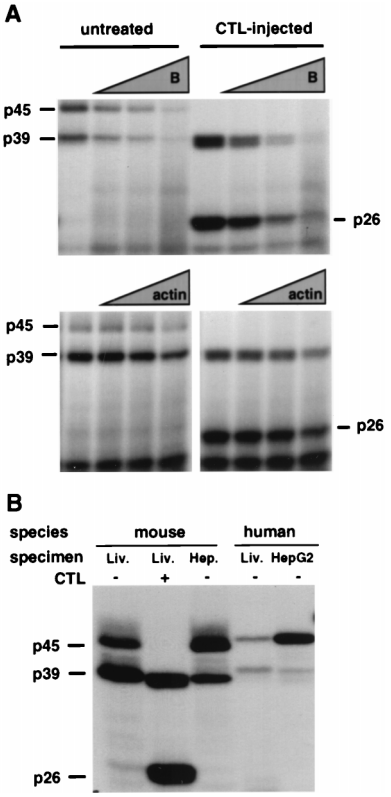

FIG. 4. (A) p45, p39, and p26 bind specifically to HBV RNA-B. A 10-, 30-, or 60-fold molar excess of unlabeled in vitro RNA-B (upper panel, lanes 2 to 4 and 6 to 8) or 500-, 1,000-, or 1,500-fold molar excess of unlabeled mouse β-actin in vitro transcript (114 nt) (lower panel, lanes 2 to 4 and 6 to 8) was added to 1 μg of liver nuclear extract from untreated or CTL-injected mice in binding buffer just before addition of 40 fmol of [^{32}P]UTP-labeled in vitro RNA-B. After irradiation, samples were RNase treated and analyzed by SDS-PAGE as described in Materials and Methods. (B) HBV RNA-binding proteins are present in mouse and human hepatocytes. Liver nuclear extracts (Liv.; 2 μg) from untreated HBV transgenic mice and CTL-injected HBV transgenic mice, nuclear extracts from partially (60%) purified hepatocytes prepared from untreated HBV transgenic mice (Hep; 1 μg), and nuclear extracts prepared from human liver (1 μg) and the human hepatocyte cell line HepG2 (1 μg) were used in the standard UV cross-linking analysis as described in Materials and Methods.

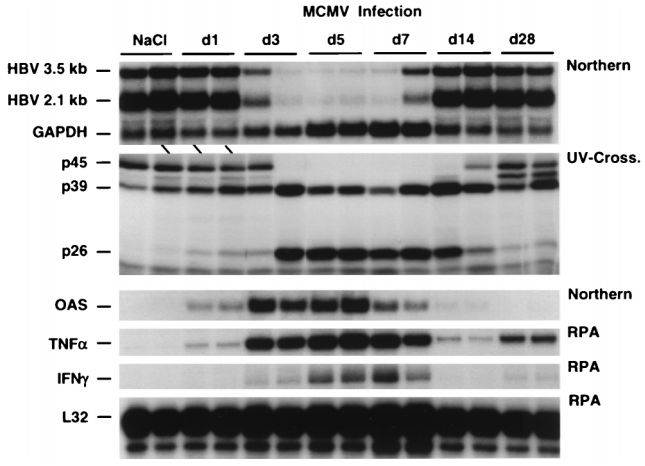

FIG. 5. Kinetics of p45, p39, and p26 binding activities during MCMV infection. HBV transgenic mice were infected with MCMV, and livers were harvested from groups of two mice sacrificed on days 1 (d1), 3, 5, 7, 14, and 28 after infection, as indicated. Total hepatic RNA and liver nuclear extracts were prepared and then analyzed by Northern blotting, UV cross-linking (UV-Cross.), and RNase protection assay (RPA) as described in Materials and Methods. Northern blots were probed for the expression of HBV RNA, GAPDH mRNA, and 2′,5′-OAS mRNA and compared with total liver RNA prepared from two saline-injected animals. Nuclear extract (5 μg) from each mouse was incubated with 40 fmol in vitro RNA-B, processed, and analyzed by SDS-PAGE. Total RNA (10 μg) from the same livers was analyzed by RNase protection assay for the expression of TNF-α and IFN-γ. The mRNA encoding the ribosomal protein L32 was used to normalize the amount of RNA loaded in each lane.

Temporal association between the disappearance of p45, the appearance of p26, and the clearance of HBV RNA after MCMV infection. We have previously reported that hepatic HBV gene expression is inhibited in the liver of lineage 1.3.32 transgenic mice during MCMV infection (8). We took advantage of this model to monitor the HBV RNA-binding proteins in liver nuclear extracts at multiple time points after MCMV infection in order to examine the temporal relationship between the induction of cytokines, the RNA-binding protein profile, and the disappearance of HBV RNA.

As shown in Fig. 5, on the first day after MCMV infection, the intrahepatic content of HBV RNA, p45, and p39 was unchanged and p26 was slightly induced. At this time, traces of TNF-α and 2′,5′-oligoadenylate synthase (2′,5′-OAS) mRNA, a marker for IFN-α/β induction, were detectable in the liver. Three days after MCMV infection, however, the intrahepatic HBV RNA content was variably decreased in the two mice studied, corresponding with the induction of intrahepatic IFN-γ, TNF-α, and 2′,5′-OAS mRNA and a commensurate reduction in p45 and induction of p26 that correlated with the amount of HBV RNA remaining in the liver. Again, the signal intensity and electrophoretic mobility of p39 were slightly increased. These changes were most pronounced on day 5, returning to baseline between 7 and 28 days after infection, demonstrating a temporal correlation between the disappearance of HBV RNA, the disappearance of p45, and the appearance of p26 during MCMV infection. The HBV RNA-binding proteins returned to baseline more slowly than the HBV RNA, however, suggesting that additional processes might control HBV gene expression late in the infection.

To further examine the correlation between intrahepatic HBV RNA content and the relative abundance of p45 and p26, we compared these parameters in livers from transgenic mice under a variety of experimental conditions which we have previously shown to either suppress HBV RNA content (CTL injection, MCMV infection, and LCMV infection [8, 25, 27]) or have little or not effect on hepatic HBV RNA content (IL-12 injection and adenovirus infection [8, 9]). As shown in Fig. 6, intrahepatic HBV RNA content was greatly reduced 5 days after the mice received HBsAg-specific CTLs or were infected by MCMV or LCMV. In each case, this was associated with the disappearance or reduction of p45 and the appearance of p26. In contrast, the HBV RNA-binding proteins were relatively unchanged after IL-12 injection or adenovirus infection, except in the one adenovirus-infected mouse that demonstrated a significant reduction in hepatic HBV RNA and a corresponding increase in the cytokine mRNAs (Fig. 6). Collectively, these results suggest a relationship between the disappearance of HBV RNA and the coordinated change in the RNA binding activities of p45 and p26. Since antibodies to TNF-α and IFN-γ can block both the downregulation of HBV RNA (27) and the modulation of p45 and p26 following CTL injection, (Fig. 2, lanes 3 to 5), these two cytokines are obviously necessary for these effects to occur. They are not sufficient, however, since they are induced to similar degrees under conditions (IL-12 injection and adenovirus infection [8, 9]) that cause little or no decrease in HBV RNA or any change in the HBV RNA-binding proteins and under conditions that abolish HBV RNA and change the balance of the RNA-binding proteins in the liver (e.g., CTL injection, LCMV infection, and MCMV infection). Apparently other, currently unknown factors must cooperate with the cytokines for these antiviral regulatory effects to occur.

DISCUSSION

We know from previous studies that TNF-α and IFN-γ downregulate HBV gene expression in the liver of HBV transgenic mice by posttranscriptionally destabilizing the viral mRNA (27, 29, 60). In this report, we demonstrate a correlation between the presence of a 45-kDa HBV RNA-binding protein in liver nuclear extracts and the presence of HBV RNA in three lineages of HBV transgenic mice. In addition, we show that the disappearance of HBV RNA is associated with the appearance of a 26-kDa HBV RNA-binding protein in liver nuclear exracts, coincident with the disappearance of p45. These changes were induced by several independent proinflammatory stimuli, including HBsAg-specific CTLs, MCMV infection, and LCMV infection, all of which downregulate intrahepatic HBV RNA

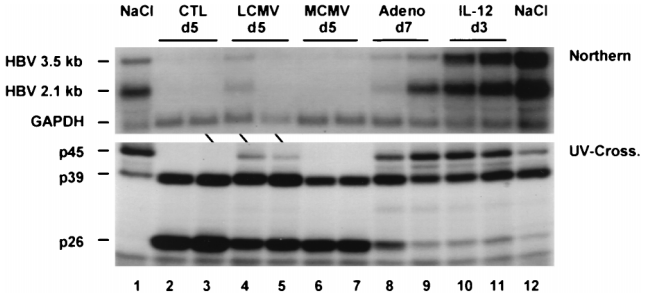

FIG. 6. RNA binding activities of p45, p39, and p26 after CTL and IL-12 injection and after MCMV, LCMV, and adenovirus infection. Groups of sex- and serum HBeAg-matched transgenic mice (two mice per group) were injected with saline or CTL and sacrificed on day 5 after injection (lineage 1.3.32; NaCl or CTL d5), infected with LCMV and sacrificed on day 5 after infection (lineage 1.3.32; LCMV d5), infected with MCMV and sacrificed on day 5 after infection (lineage 1.3.32; (MCMV d5), infected with adenovirus and sacrificed on day 7 after infection (lineage 1.3.32; Adeno d7), or injected with IL-12 daily for 3 consecutive days and sacrificed on 1 day after the third injection (IL-12 d3). Total hepatic RNA and liver nuclear extracts were prepared and analyzed as described in the legend to Fig. 5.

(8, 25, 27). Furthermore, we demonstrate that all three proteins interact specifically with RNA-B and that the disappearance of p45 and the appearance of p26 are mediated by TNF-α and IFN-γ. Collectively, these results suggest that the RNA-binding proteins described in this report might be components of a cytokine-inducible signal transduction pathway that regulates HBV RNA stability. Proof of this hypothesis will require the identification, cloning, and functional characterization of these proteins in future experiments. *trans*-acting factors such as RNA-binding proteins and RNases are known to interact with sequence and/or structural elements in RNA to mediate the splicing, nuclear export, degradation, and stabilization of cellular and viral transcripts. Accordingly, the steady-state content of HBV RNA in a cell might be regulated by the coordinated activity of stabilizing and destabilizing RNA-binding proteins, RNases, and the nuclear export machinery. The nuclear export of HBV RNA is thought to be mediated by a previously described PRE, located between nt 1239 and 1805 (32, 33), which targets the HBV RNA to a splicing-independent export pathway (18, 32). Indeed, it was the fact that the 5′ terminus of the PRE is located within the 3.5-, 2.4-, and 2.1-kb HBV transcripts that are downregulated by the inflammatory cytokines and is absent from the 0.7-kb mRNA which is resistant to cytokine-induced downregulation that stimulated us to focus on this region to search for the HBV RNA-binding proteins described in this report.

Two cellular proteins (p30 and p45) that bind to PRE region III (nt 1487 to 1582) have been previously identified (34). However, since these proteins do not bind to PRE region I (nt 1239 to 1375), which contains the 91-nt element (nt 1243 to 1333) used in our experiments, they are probably not related to the HBV RNA-binding proteins described herein. It is theoretically possible that HBV RNA-binding proteins such as p45 can protect HBV RNA from a rate-limiting cleavage event by binding to a potential cleavage site. This scenario has been proposed for transferrin receptor mRNA, which is protected against endonucleolytic cleavage by an RNA IRE-binding cellular protein (4). This hypothesis is supported by our recent identification of an endonucleolytic cleavage site within RNA-B and full-length HBV RNA obtained from HBV transgenic mouse liver and by preliminary data indicating that this cleavage is more efficient in p45-deficient liver nuclear extracts (30a).

Obviously, until these HBV RNA-binding proteins are identified and their functional roles in the stabilization and/or destabilization of HBV RNA can be directly tested, we must consider the possibility that they are related to known RNA-binding proteins that regulate the stability of other transcripts. For example, AUF1 consists of a family of isoforms, including several whose molecular masses (37, 40, 42, and 45 kDa) are similar to those of p45 and p39 (37). Similarly, poly(rC)-binding protein 2 (PCBP-2), which is required for the translation of poliovirus RNA (5) and globin mRNA stabilization (62), has a molecular mass of around 39 kDa. Since the binding of p45 and p39 to RNA-B could be competed by poly(rU) and poly(rG) and not by poly(rC) (29a), it seems likely that p45 is different from AUF1, since AUF1 cannot be competed by poly(G) (6), and from PCBP. Furthermore, p45 and p39 are similar in size to hnRNP-D (44 to 48 kDa), hnRNP-G (43 kDa), hnRNP-E (36 to 43 kDa), hnRNP-B2 (39 kDa), and hnRNPA2/B1 (36/38 kDa) (19). Also, p45, p39, and p26 are similar in size to the full-length and proteolytic products of the RNA-binding protein SS-B/La detected in calf thymus extracts (10, 11).

Characterization of the RNA binding activities revealed that RNA-protein complex formation was detectable under low ionic strength but the RNA binding activity of p45, p39, and p26 was more efficient at high salt concentrations (Fig. 3B); during the formation of other RNA-protein complexes, in contrast, the binding affinity usually decreases with increasing ionic strength (7, 30, 61, 63), presumably due to competition between the salt anions and the nucleic acid anions for protein-binding sites (59). The preference of p45, p39, and p26 for high salt concentrations suggests that nonelectrostatic (i.e., hydrophobic) interactions may contribute to their binding affinity for RNA-B. It remains to be determined whether the binding reaction at high ionic strength has some functional correlate under physiological salt concentrations; nonetheless, the binding of p45, p39, and p26 to RNA-B was clearly observed at low salt concentrations (Fig. 3B).

We do not know whether p45 and p26 bind HBV RNA individually or as a complex with p39. However, results of UV cross-linking under nonreducing conditions showed that p45, p39, and p26 are not covalently bound to each other (Fig. 3A). Furthermore, the detection of p39 in the absence of p45 in nuclear extracts from spleen and lung suggests that all three proteins can bind HBV RNA independently (29a). Globin mRNA stability is regulated by a protein complex composed of at least two PCBPs, AUF1 proteins, and additional unidentified proteins (36), showing that in some cases the regulation of mRNA degradation depends on the coordinated activity of several RNA-binding proteins. Analysis of RNA-protein complexes eluted from a native polyacrylamide gel and fractionated by sucrose gradients is needed to elucidate the nature of the native HBV RNA-protein complexes that are present in nuclear extracts from untreated and CTL-injected HBV transgenic mice.

We have shown previously that MCMV and LCMV infection of HBV transgenic mice can inhibit HBV gene expression and replication (8, 25), while IL-12 injection and adenovirus infection abolish HBV replication without a commensurate decrease in HBV gene expression (8, 9, 25). We have also shown that both effects can be blocked by antibodies against TNF-α and IFN-γ (reference 27 and this study) and that the downregulation of HBV replication after LCMV, MCMV, and adenovirus infection can also be blocked by antibodies against IFN-α/β (8, 25). These results indicate that the induction of TNF-α, IFN-γ, and probably IFN-α/β are essential but not sufficient for the destabilization of HBV RNA and the modulation of the RNA-binding activity of p45 and p26.

It is well established that cytokines can affect the stability of certain mRNAs, including viral RNA. The best-characterized cytokine-induced antiviral mechanisms are the induction of double-stranded RNA-regulated protein kinase (PKR) by IFN-γ and of 2′,5′-OAS by IFN-α/β. Both PKR and 2′,5′-OAS need double-stranded RNA to be activated. The production of 2′,5′-adenylates results in the activation of RNase L, which degrades single-stranded RNA. Since 2′,5′-OAS mRNA is rapidly induced in the mice following CTL injection and MCMV and LCMV infection (not shown), it is possible that RNase L degrades HBV RNA. However, additional experiments will be needed to determine if PKR or RNase L contributes to the posttranscriptional downregulation of HBV RNA in our models.

In addition, it has been shown that IFN-γ can selectively downregulate c-*fos* mRNA (47), CD23/FcRII mRNA (39), and cystic fibrosis transmembrane conductance regulator mRNA (3) and that it can stabilize complement components C3 and C4 mRNAs (43) and intercellular adhesion molecule 1 mRNA in murine fibroblasts (45) at the posttranscriptional level. Furthermore, TNF-α is known to destabilize GLUT-4 (40) and surfactant protein B (46) mRNAs and to stabilize GLUT-1 (58) and IL-1 (24) mRNAs. These results show that the same cytokine can posttranscriptionally destabilize or stabilize viral

and cellular mRNAs, providing precedent for the results presented in this report.

Obviously, overexpression or deletion of these proteins will be necessary to define their functional potential. Similarly, proof of the stabilizing and/or destabilizing activity of the 91-nt RNA element described here requires analysis of the influence of the element on the abundance of a reporter transcript of known stability. Since we have not been able to reproduce the antiviral effect of the cytokines in vitro, definitive experiments to determine whether HBV RNA stability is regulated by the family of HBV RNA-binding proteins described in this report will have to be performed in vivo.

ACKNOWLEDGMENTS

We thank Robert Schreiber (Washington University, St. Louis, Mo.) for providing the hamster monoclonal antibody to murine IFN-γ and TNF-α; Maurice Gately (Hoffman La Roche, Nutley, N.J.) for providing IL-12; James Wilson (University of Pennsylvania, Philadelphia) for providing the recombinant adenovirus Ad.CBlacZ; Ann Campbell (Eastern Virginia Medical School, Norfolk, Va.) for providing MCMV; Monte Hobbs (University of Michigan, Ann Arbor) for providing the cytokine gene and T-cell marker probe sets used in the RNase protection assays; Thomas J. Hope (Salk Institute, La Jolla, Calif.) for providing plasmids carrying the HIV RRE and Mason-Pfizer virus CRL elements; the Scripps Molecular Biology Core Facility for the production of oligonucleotides; Rick Koch for excellent technical assistance; and Jennifer Newmann and Pamela Fagan for help with manuscript preparation.

This work was supported by grants R37-CA40489 and R01-AI40696 from the National Institutes of Health.

REFERENCES

1. **Alonso, S., A. Minty, Y. Bourlet, and M. Buckingham.** 1986. Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. J. Mol. Evol. **23:**11–22.
2. **Ando, K., L. G. Guidotti, A. Cerny, T. Ishikawa, and F. V. Chisari.** 1994. CTL access to tissue antigen is restricted in vivo. J. Immunol. **153:**482–488.
3. **Besancon, F., G. Przewlocki, I. Baro, A. S. Hongre, D. Escande, and A. Edelman.** 1994. Interferon-gamma downregulates CFTR gene expression in epithelial cells. Am. J. Physiol. **267:**C1398–C1404.
4. **Binder, R., J. A. Horowitz, J. P. Basilion, D. M. Koeller, R. D. Klausner, and J. B. Harford.** 1994. Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3′ UTR and does not involve poly(A) tail shortening. EMBO J. **13:**1969–1980.
5. **Blyn, L. B., K. M. Swiderek, O. Richards, D. C. Stahl, B. L. Semler, and E. Ehrenfeld.** 1996. Poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus RNA 5′ noncoding region: identification by automated liquid chromatography-tandem mass spectrometry. Proc. Natl. Acad. Sci. USA **93:**11115–11120.
6. **Brewer, G.** 1991. An A+U-rich element RNA-binding factor regulates c-*myc* mRNA stability in vitro. Mol. Cell. Biol. **11:**2460–2466.
7. **Carey, J., V. Cameron, P. L. de Haseth, and O. C. Uhlenbeck.** 1983. Sequence-specific interaction of R17 coat protein with its ribonucleic acid binding site. Biochemistry **22:**2601–2610.
8. **Cavanaugh, V. J., L. G. Guidotti, and F. V. Chisari.** 1998. Inhibition of hepatitis B virus replication during adenovirus and cytomegalovirus infections in transgenic mice. J. Virol. **72:**2630–2637.
9. **Cavanaugh, V. J., L. G. Guidotti, and F. V. Chisari.** 1997. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. J. Virol. **71:**3236–3243.
10. **Chan, E. K., A. M. Francoeur, and E. M. Tan.** 1986. Epitopes, structural domains, and asymmetry of amino acid residues in SS-B/La nuclear protein. J. Immunol. **136:**3744–3749.
11. **Chan, E. K., K. F. Sullivan, and E. M. Tan.** 1989. Ribonucleoprotein SS-B/La belongs to a protein family with consensus sequences for RNA-binding. Nucleic Acids Res. **17:**2233–2244.
12. **Chen, C. Y., and A. B. Shyu.** 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20:**465–470.
13. **Chen, C. Y., and A. B. Shyu.** 1994. Selective degradation of early-response-gene mRNAs: functional analyses of sequence features of the AU-rich elements. Mol. Cell. Biol. **14:**8471–8482.
14. **Chisari, F. V.** 1997. Cytotoxic T cells and viral hepatitis. J. Clin. Investig. **99:**1472–1477.
15. **Chisari, F. V., and C. Ferrari.** 1995. Hepatitis B virus immunopathology. Springer Semin. Immunopathol. **17:**261–281.
16. **Chisari, F. V., C. A. Pinkert, D. R. Milich, P. Filippi, A. McLachlan, R. D. Palmiter, and R. L. Brinster.** 1985. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. Science **230:**1157–1160.
17. **Decker, C. J., and R. Parker.** 1994. Mechanisms of mRNA degradation in eukaryotes. Trends Biochem. Sci. **19:**336–340.
18. **Donello, J. E., A. A. Beeche, G. J. Smith, G. R. Lucero, and T. J. Hope.** 1996. The hepatitis B virus posttranscriptional regulatory element is composed of two subelements. J. Virol. **70:**4345–4351.
19. **Dreyfuss, G., M. J. Matunis, S. Pinol-Roma, and C. G. Burd.** 1993. hnRNP proteins and the biogenesis of mRNA. Annu. Rev. Biochem. **62:**289–321.
20. **Eisenstein, R. S., P. T. Tuazon, K. L. Schalinske, S. A. Anderson, and J. A. Traugh.** 1993. Iron-responsive element-binding protein. Phosphorylation by protein kinase C. J. Biol. Chem. **268:**27363–27370.
21. **Fridell, R. A., H. P. Bogerd, and B. R. Cullen.** 1996. Nuclear export of late HIV-1 mRNAs occurs via a cellular protein export pathway. Proc. Natl. Acad. Sci. USA **93:**4421–4424.
22. **Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature **281:**646–650.
23. **Gilles, P. N., G. Fey, and F. V. Chisari.** 1992. Tumor necrosis factor alpha negatively regulates hepatitis B virus gene expression in transgenic mice. J. Virol. **66:**3955–3960.
24. **Gorospe, M., S. Kumar, and C. Baglioni.** 1993. Tumor necrosis factor increases stability of interleukin-1 mRNA by activating protein kinase C. J. Biol. Chem. **268:**6214–6220.
25. **Guidotti, L. G., P. Borrow, M. V. Hobbs, B. Matzke, I. Gresser, M. B. Oldstone, and F. V. Chisari.** 1996. Viral cross talk: intracellular inactivation of the hepatitis B virus during an unrelated viral infection of the liver. Proc. Natl. Acad. Sci. USA **93:**4589–4594.
26. **Guidotti, L. G., S. Guilhot, and F. V. Chisari.** 1994. Interleukin-2 and alpha/beta interferon down-regulate hepatitis B virus gene expression in vivo by tumor necrosis factor-dependent and -independent pathways. J. Virol. **68:**1265–1270.
27. **Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber, and F. V. Chisari.** 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity **4:**25–36.
28. **Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari.** 1995. High-level hepatitis B virus replication in transgenic mice. J. Virol. **69:**6158–6169.
29. **Guilhot, S., L. G. Guidotti, and F. V. Chisari.** 1993. Interleukin-2 downregulates hepatitis B virus gene expression in transgenic mice by a posttranscriptional mechanism. J. Virol. **67:**7444–7449.

29a. **Heise, T.** Unpublished data.

30. **Heise, T., A. Nath, K. Jungermann, and B. Christ.** 1997. Purification of a RNA-binding protein from rat liver. Identification as ferritin L chain and determination of the RNA/protein binding characteristics. J. Biol. Chem. **272:**20222–20229.

30a. **Heise, T., et al.** Unpublished data.

31. **Hentze, M. W., and L. C. Kuhn.** 1996. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc. Natl. Acad. Sci. USA **93:**8175–8182.
32. **Huang, J., and T. J. Liang.** 1993. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. Mol. Cell. Biol. **13:**7476–7486.
33. **Huang, Z. M., and T. S. Yen.** 1994. Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts in the cytoplasm. J. Virol. **68:**3193–3199.
34. **Huang, Z. M., W. Q. Zang, and T. S. Yen.** 1996. Cellular proteins that bind to the hepatitis B virus posttranscriptional regulatory element. Virology **217:**573–581.
35. **Izaurralde, E., and I. W. Mattaj.** 1995. RNA export. Cell **81:**153–159.
36. **Kiledjian, M., C. T. DeMaria, G. Brewer, and K. Novick.** 1997. Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the alpha-globin mRNA stability complex. Mol. Cell. Biol. **17:**4870–4876.
37. **Kiledjian, M., X. Wang, and S. A. Liebhaber.** 1995. Identification of two KH domain proteins in the alpha-globin mRNP stability complex. EMBO J. **14:**4357–4364.
38. **Kozarsky, K. F., and J. M. Wilson.** 1993. Gene therapy: adenovirus vectors. Curr. Opin. Genet. Dev. **3:**499–503.
39. **Lee, C. E., S. R. Yoon, and K. H. Pyun.** 1993. Mechanism of interferon-gamma down-regulation of the interleukin 4-induced CD23/Fc epsilon RII expression in human B cells: post-transcriptional modulation by interferon-gamma. Mol. Immunol. **30:**301–307.
40. **Long, S. D., and P. H. Pekala.** 1996. Regulation of GLUT4 mRNA stability by tumor necrosis factor-alpha: alterations in both protein binding to the 3′ untranslated region and initiation of translation. Biochem. Biophys. Res. Commun. **220:**949–953.
41. **Malter, J. S.** 1989. Identification of an AUUUA-specific messenger RNA binding protein. Science **246:**664–666.
42. **Melefors, O., and M. W. Hentze.** 1993. Iron regulatory factor—the conductor of cellular iron regulation. Blood Rev. **7:**251–258.
43. **Mitchell, T. J., M. Naughton, P. Norsworthy, K. A. Davies, M. J. Walport, and B. J. Morley.** 1996. IFN-gamma up-regulates expression of the comple-

ment components C3 and C4 by stabilization of mRNA. J. Immunol. **156:** 4429–4434.
44. **Moriyama, T., S. Guilhot, K. Klopchin, B. Moss, C. A. Pinkert, R. D. Palmiter, R. L. Brinster, O. Kanagawa, and F. V. Chisari.** 1990. Immunobiology and pathogenesis of hepatocellular injury in hepatitis B virus transgenic mice. Science **248:**361–364.
45. **Ohh, M., and F. Takei.** 1994. Interferon-gamma- and phorbol myristate acetate-responsive elements involved in intercellular adhesion molecule-1 mRNA stabilization. J. Biol. Chem. **269:**30117–30120.
46. **Pryhuber, G. S., S. L. Church, T. Kroft, A. Panchal, and J. A. Whitsett.** 1994. 3′-untranslated region of SP-B mRNA mediates inhibitory effects of TPA and TNF-alpha on SP-B expression. Am. J. Physiol. **267:**L16–L24.
47. **Radzioch, D., and L. Varesio.** 1991. c-*fos* mRNA expression in macrophages is downregulated by gamma interferon at the posttranscriptional level. Mol. Cell. Biol. **11:**2718–2722.
48. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:**423–450.
49. **Russell, J. E., J. Morales, and S. A. Liebhaber.** 1997. The role of mRNA stability in the control of globin gene expression. Prog. Nucleic Acid Res. Mol. Biol. **57:**249–287.
50. **Sabath, D. E., H. E. Broome, and M. B. Prystowsky.** 1990. Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. Gene **91:**185–191.
51. **Schaller, H., and M. Fischer.** 1991. Transcriptional control of hepadnavirus gene expression. Curr. Top. Microbiol. Immunol. **168:**21–39.
52. **Schoenberg, D. R., and E. Chernokalskaya.** 1997. Ribonucleases involved in eukaryotic mRNA turnover, p. 217–240. *In* J. B. Harford and D. R. Morris (ed.), mRNA metabolism and post-transcriptional gene regulation. Wiley-Liss, New York, N.Y.
53. **Schreiber, R. D., L. J. Hicks, A. Celada, N. A. Buchmeier, and P. W. Gray.** 1985. Monoclonal antibodies to murine gamma-interferon which differentially modulate macrophage activation and antiviral activity. J. Immunol. **134:**1609–1618.
54. **Sharp, P. A.** 1994. Split genes and RNA splicing. Cell **77:**805–815.
55. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
56. **Sheehan, K. C., N. H. Ruddle, and R. D. Schreiber.** 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J. Immunol. **142:**3884–3893.
57. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosuppressive D-type retrovirus. Cell **45:**375–385.
58. **Stephens, J. M., B. Z. Carter, P. H. Pekala, and J. S. Malter.** 1992. Tumor necrosis factor alpha-induced glucose transporter (GLUT-1) mRNA stabilization in 3T3-L1 preadipocytes. Regulation by the adenosine-uridine binding factor. J. Biol. Chem. **267:**8336–8341.
59. **Talbot, S. J., and S. Altman.** 1994. Kinetic and thermodynamic analysis of RNA-protein interactions in the RNase P holoenzyme from Escherichia coli. Biochemistry **33:**1406–1411.
60. **Tsui, L. V., L. G. Guidotti, T. Ishikawa, and F. V. Chisari.** 1995. Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes. Proc. Natl. Acad. Sci. USA **92:**12398–12402.
61. **Vartikar, J. V., and D. E. Draper.** 1989. S4-16 S ribosomal RNA complex. Binding constant measurements and specific recognition of a 460-nucleotide region. J. Mol. Biol. **209:**221–234.
62. **Wang, X., M. Kiledjian, I. M. Weiss, and S. A. Liebhaber.** 1995. Detection and characterization of a 3′ untranslated region ribonucleoprotein complex associated with human alpha-globin mRNA stability. Mol. Cell. Biol. **15:** 1769–1777.
63. **Weeks, K. M., and D. M. Crothers.** 1992. RNA binding assays for Tat-derived peptides: implications for specificity. Biochemistry **31:**10281–10287.